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The Gut Microbiome as a Mediator of Type 2 Diabetes Mellitus in Mexican Americans

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The Microbiome as a Mediator of Type 2 Diabetes Mellitus in Mexican Americans

by

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Dedication

I dedicate this thesis to my parents, Derek and Jane Kitten, who have supported me in every way over the last 28 years; to my sister, Natalie Marshall, for taking care of me when I most needed it; and to Isaac Perales, for his unwavering encouragement and love.

Acknowledgements

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Abstract

The Microbiome as a Mediator of Type 2 Diabetes Mellitus in Mexican Americans

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The University of Texas at Austin, 2019

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Type 2 diabetes mellitus (T2DM) is an urgent public health problem and disproportionately affects Mexican-Americans. The gut microbiome contributes to the pathophysiology of diabetes; however, no studies have examined this association in Mexican-Americans. The objective of this study was to compare gut microbiome composition between Mexican-Americans with and without T2DM.

This was a cross-sectional study of volunteers from San Antonio, TX. Subjects were 18 years or older and self-identified as Mexican-American. Subjects were grouped by T2DM diagnosis. Eligible subjects attended a clinic visit to provide demographic and medical information. Thereafter, subjects recorded what they ate for three days and collected a stool sample on the fourth day. Stool 16s rRNA sequences were classified into operational taxonomic units (OTUs) via Mothur's Bayesian classifier and referenced to the Greengenes database. Alpha diversity and taxa relative abundance were compared between groups using the Wilcoxon rank sum test. Beta diversity was estimated using Bray-Curtis indices and compared between groups using PERMANOVA.

Thirty-seven subjects were included, 14 (38%) with diabetes and 23 (62%) without diabetes. Groups were well-matched by body mass index (BMI) (diabetes 30 mg/kg2, no diabetes 28 mg/kg2; p=0.4653) and other comorbid conditions. Alpha diversity was not significantly different between those with and without T2DM (3.21 vs. 3.07; p=0.3409). Beta diversity was not significantly associated with T2DM diagnosis (p=0.1249). Sixteen operational taxonomic units (OTUs) were significantly different between groups. There was a significantly lower relative abundance (RA) of *Streptococcus* in those with T2DM (p=0.04). The Firmicutes to Bacteroidetes ratio was higher in those with T2DM (0.637:1) compared to those without T2DM (0.507:1).

In conclusion, although alpha diversity was not different between diabetic and non-diabetic Mexican-Americans, the microbial composition was significantly different.

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Chapter One: Role of the Microbiome in Human Health

Overview of the Human Microbiome

The human microbiome is an expanding area of clinical research. Scientific literature examining the role of the microbiome in human health has expanded substantially over the last ten years.[1] This has led to the identification of the microbiome as a major contributor to human health and disease.[2] The microbiome refers to the collection of all genomes of microbes in an ecosystem. On the other hand, microbiota describes the microbes, including bacteria, archea, viruses, and fungi, that collectively inhabit a given ecosystem.[3] Dysbiosis occurs when there is a disturbance or change in the composition and function of these microbes. Many studies focus specifically on the bacterial component of the gut microbiota, as bacteria contribute the greatest amount of genetic material (approximately 99.1%, compared to 0.1% of genetic material from eukaryotic and viral sources).[4] The impact of the human microbiome on health is not surprising considering its scope; the number of bacterial cells outnumber human cells at a ratio of 10:1.[5] Of the vast number of bacterial cells in and on the human body, 95% are located in the gastrointestinal tract.

Several tools exist for the purposes of analyzing the contents of the microbiome (Figure 1). Different tools can be used to identify community composition, gene expression, protein expression, and metabolic activity.[6] The most common approach is 16S rRNA gene sequencing which utilizes 16S rRNA amplification with polymerase chain reaction (PCR). These genes are then sequenced using next-generation sequencing technology. Machine learning is then used to cluster similar sequences, and reference databases, such as Greengenes, assist with assigning taxonomy.

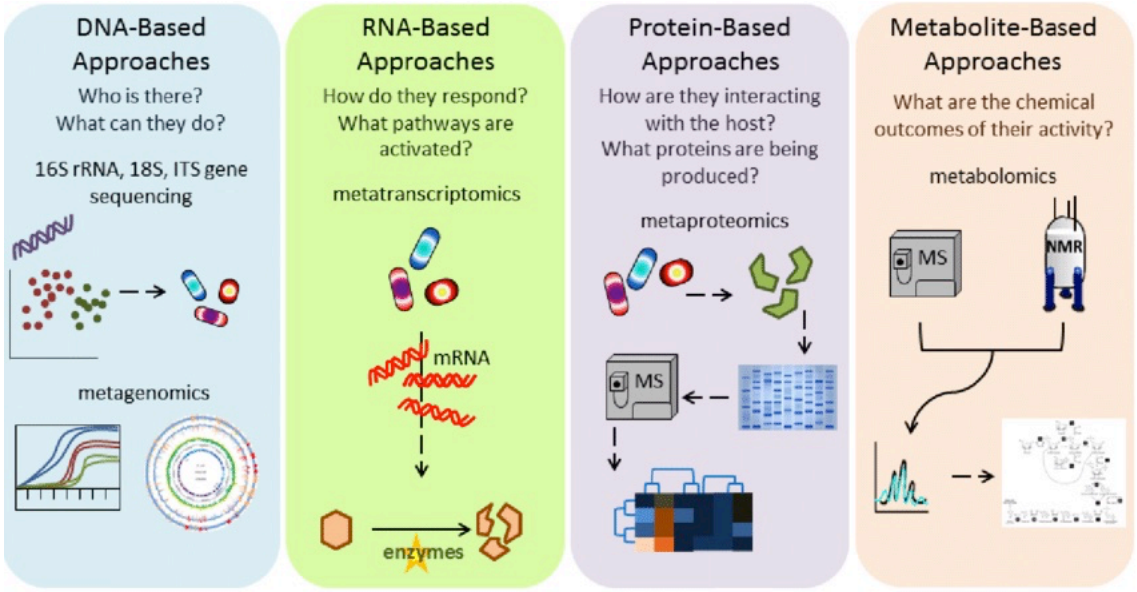

Figure 1: Gut Microbiome Analysis (adapted from Ishiguro and Campbel 2018)[7]

The composition of the human microbiome varies by body site. Outer body sites are predominated by Gram-positive aerobic organisms from the Actinobacteria and Firmicutes phyla.[8] The gut microbiome is predominated by anaerobic Gram-positive and Gram-negative bacteria, including the phyla Firmicutes, Bacteroidetes, and Actinobacteria. Most variation in the microbiota occurs at progressively lower taxonomic levels, with high conservation at the highest levels.[8]

Several measures exist to aid in comparing the gut microbiomes of different groups. First, alpha diversity measures the quantity of different bacterial taxa, or richness, within a bacterial community.[9] High alpha diversity indicates a high quantity of different bacteria. Shannon diversity, a type of alpha diversity, accounts for both richness and evenness, where evenness refers to the degree of similarity in the proportional abundance between different bacterial taxa. Beta diversity refers to differences in diversity between subjects.

Beta diversity is visually represented using a principle coordinate analysis (PCoA). To produce a PCoA, each bacterial community is assigned two coordinates based on compositional diversity. Each community is then plotted on a diagram where distance between points represents the overall difference between the communities.

Global Gut Microbiota Functions

The gut microbiome carries out several important functions. It helps to train the immune system and inhibits invasion by pathogens, such as *Clostridioides difficile*.[3,10] The gut microbiota also mediates host-cell proliferation and vascularization and regulates multiple signaling molecules that control endocrine and neurologic function.[3] Gut bacteria also provide a source of energy biosynthesis. Additionally, they synthesize vitamins, neurotransmitters, and other related compounds. Other important roles include metabolism of bile salts and xenobiotic metabolism and elimination.

Associations Between Gut Dysbiosis and Human Disease

Given the many functions of the gut microbiome, it is no surprise that an association exists between gut dysbiosis and human disease.[2] Multiple factors influence the composition of the gut microbiome, those which are not modifiable, including neonatal mode of delivery, host genetic features, host immune response, and age, as well as those that are modifiable: diet, medications, environmental exposures, physical activity, smoking, and alcohol consumption.[3] An imbalance in the gut community can cause a shift from a healthy metabolic condition to one that predisposes an individual to disease

development including diabetes, obesity, metabolic syndrome, cancer, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and cardiovascular disease (Table 1).

Table 1: Influence of the Gut Microbiome on Health

Health	**Microbial products or activities**	**Disease**
Nutrient & energy supply	• SCFA production & vitamin synthesis • Energy supply, gut hormones, & satiety • Lipopolysaccharides, inflammation	Obesity & metabolic syndrome
Cancer prevention	• Butyrate production, phytochemical release • Toxin and carcinogen inflammation • Mediates inflammation	Cancer promotion
Pathogen inhibition	• SCFA production, intestinal pH, bacteriocins • Competition for substrates and/or binding sites • Toxin production, tissue invasion, inflammation	Pathogen invasion
GI immune function	• Balance of pro- and anti-inflammatory signals • Inflammation, immune disorders	IBD
Gut motility	• Metabolites (SCFAs, gases) from non-digestible carbohydrates	IBS (constipation, diarrhea, bloating)
Cardiovascular health	• Lipid & cholesterol metabolism	Cardiovascular disease

GI, gastrointestinal; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome

Chapter Two: Gut Dysbiosis and Diabetes Mellitus

Overview of Diabetes Mellitus

As of 2015, 30.3 million Americans, or 9.4% of the population, had diabetes mellitus (DM).[11] Approximately one-third were undiagnosed. The majority of those with diabetes (approximately 29 million of the 30.3 million have T2DM). Diabetes represents a substantial burden and is associated with significant morbidity and mortality.[12,13] In 2017, diabetes and its complications, including cardiovascular disease, retinopathy, nephropathy, and neuropathy, resulted in an annual cost of $237 billion in direct medical costs and $90 billion in reduced productivity.[11] In 2015 diabetes was the seventh leading cause of death in the United States.

Pathophysiology: The Egregious Eleven

Healthcare providers' understanding of diabetes has evolved over time. Although diabetes was initially understood as a disorder of the pancreas, researchers have now identified 11 dysfunctional pathways that contribute to the development of diabetes (Figure 2).[14,15]

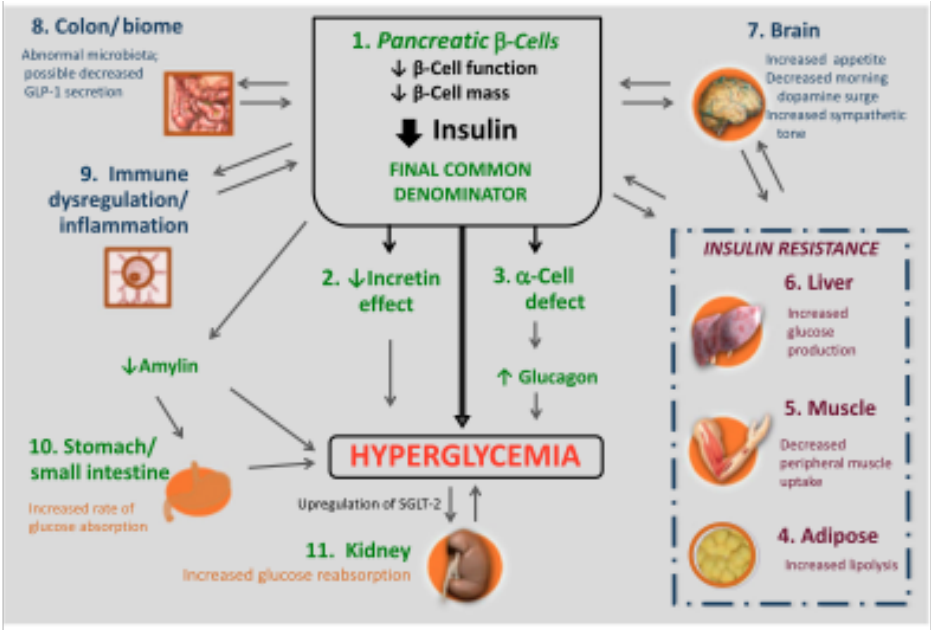

Figure 2: Beta-cell-centric Construct: Egregious Eleven (adapted from Schwartz, et al 2016)[15]

All 11 pathways contribute to diabetes pathophysiology by either negatively affecting beta-cell function or further potentiating hyperglycemia.[15] The most recently identified pathways include immune dysregulation, increased glucose absorption from the gastrointestinal tract, and changes in the gut microbiome composition.

Studies of the gut microbiome have identified major mechanisms through which it can influence energy homeostasis and contribute to diabetes (Figure 3).[16]

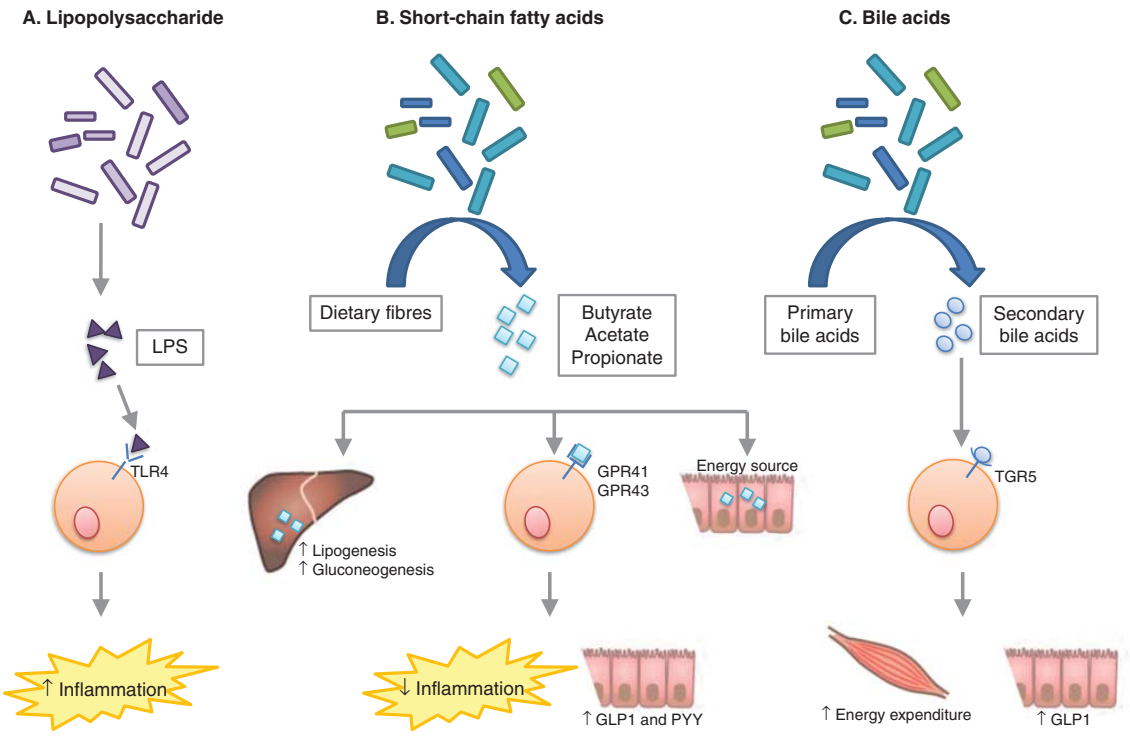

Figure 3: Microbiome and Host Metabolism (adapted from Allin, et al, 2015)[16]

First, high levels of lipopolysaccharides (LPS) exert detrimental effects on glucose homeostasis. Gram-negative bacteria shed LPS from their cell walls.[17,18] LPS bind to the toll-like receptor-4 (TLR4)/CD12 complex, which activates the innate immune system. Additionally, LPS decrease the expression of tight junction proteins. The resultant decrease in the integrity of the gut mucosa allows for translocation of LPS and intestinal microbes into the bloodstream. Studies have demonstrated that subjects with DM have higher plasma levels of LPS compared to their healthy counterparts.[18] Increased LPS levels lead to further systemic inflammation, immune cell invasion of liver and adipose tissue, and ensuing insulin resistance in these tissues.[16]

Short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate, on the other hand, have beneficial effects with respect to glucose metabolism.[18] SCFAs are produced through bacterial fermentation of non-digestible polysaccharides and serve as the main energy source for the gut epithelium and bind G-protein coupled receptors (GPCRs). Binding of SCFAs to GPCRs 41 and 43 induces glucagon-like peptide 1 (GLP-1) secretion which suppresses appetite, slows digestion, and increases insulin sensitivity. Additionally, GPCR binding inhibits inflammatory signaling molecules NF-kappa-B, tumor necrosis factor-alpha, and interleukin-6, with an overall effect of decreasing inflammation. In diabetes-associated gut dysbiosis, there is a relative deficiency of bacteria that produce SCFAs.[4,19,20]

Finally, in diabetes there is a relative deficiency of bacteria that produce bile salt hydrolases.[19,21-24] Bile salt hydrolases have the important role of converting primary bile acids to secondary bile acids. Secondary bile acids act as signaling molecules to induce GLP-1 secretion from small intestinal L-cells. Changes in the relative abundance (RA) of certain bacteria have been implicated in these major mechanisms (Table 2).

Table 2: Bacteria Involved in Metabolism

LPS Producing Bacteria	**SCFA Producers**	**Microbiota with bile salt hydrolases**
E. coli *Salmonella* *Shigella* *Pseudomonas* *Neisseria* *H. influenza* *Bordetella pertussis* *Vibrio cholerae*	*Roseburia sp.* *Faecalibacterium prausnitzii* *Eubacterium hallii* *Eubacterium rectale*	*Lactobacillus* *Bifidobacterium* Firmicutes *Enterococcus* *Clostridium* *Bacteroides*

Researchers have identified two phyla, Firmicutes and Bacteroidetes, as appreciable producers of SCFAs and LPSs, respectively.[17,18,25,26] The Firmicutes to Bacteroidetes ratio, which allows for comparison of both phyla's RA, has been positively associated with metabolic disorders, possibly due to the increased energy harvest due to high levels of SCFAs.[27]

THE GUT MICROBIOME COMPOSITION IN PATIENTS WITH DIABETES

Several studies comparing the gut microbiomes of subjects with and without diabetes have yielded varying results. The earliest microbiome studies, performed in BioBreeding rats, indicated a relationship between the microbiome and metabolic disease.[28] Specifically, alpha diversity correlated positively with higher disease rates. Lack of gut microbial diversity has been implicated in type 1 diabetes[29-31], Crohn's disease[32], colorectal cancer[33], and multiple sclerosis.[34] Surprisingly, only one of the human studies of the gut microbiome in T2DM reflected this association between lack of diversity and disease.[24] In contrast, other studies have not demonstrated any relationship between microbial diversity and T2DM,[19,22] and only one study identified inter-group differences in beta-diversity.[22]

In addition to analyzing diversity, previous studies have evaluated the abundance of various bacterial taxa. Bacteria significantly depleted in subjects with T2DM include: *Bifidobacterium* genus[22,23], Firmicutes phylum[19,21], and *Roseburia* genus.[4,19,20] The relationship between these microbes and metabolism have been described previously. In mice studies, an increase in gut *Bifidobacterium* attributable to prebiotic fiber ingestion resulted in improved glucose tolerance and decreases in inflammatory markers.[35] A high RA of Firmicutes, especially in relation to Bacteroidetes RA, has been implicated in obesity

and high BMI.[8] Interestingly, the Firmicutes phylum contains many SCFA-producing bacteria that confer metabolic benefits.[18] Specifically, many *Roseburia* species, which are members of the Firmicutes phyla, are butyrate-producers. Additionally, fecal transplantation from lean donors to subjects with metabolic syndrome resulted in an increase in *Roseburia* RA, butyrate levels, and insulin sensitivity.[36]

The bacterial taxon consistently shown to be enriched in T2DM is the *Lactobacillus* genus.[4,19,20,22,23] *Lactobacillus* has been implicated in obesity and is thought to be an immune-modulating bacteria.[37,38]

Studies have yielded conflicting results regarding the RA of *Prevotella*[19,24], *Bacteroides* [22,24], and Clostridia[19,24] in T2DM. These discordant findings are likely due to differences in study population characteristics, such as age, diet, host genotype, physical activity, and geographic location. Further studies investigating the gut microbiome composition and diabetes relationship are needed.

Table 3: Bacterial Taxa Differences in Previous Studies

References	**Microbiota in subjects with T2DM**
Wu et al. (2010), Sedighi et al. (2017)	• Lower RA of *Bifidobacterium*
Larsen et al. (2010), Lambeth et al. (2015)	• Lower RA of Firmicutes, also negatively correlated with PG values
Larsen, et al. (2010), Qin et al. (2012), Karlsson et al. (2013)	• *Roseburia* significantly depleted, negative (non-significant) correlation with plasma glucose (R=-0.53, p=0.06)
Larsen et al. (2010), Zhang et al (2013)	• Higher RA of *Prevotella* • *Bacteroides-Prevotella* to Clostridia ratio positively correlated with PG (R=0.38, p=0.03)
Wu et al. (2017)	• Lower RA of *Prevotella* (10.7% versus 58.8%; p<0.05)
Zhang et al. (2013)	• Lower *Bacteroides* RA
Wu et al.	• Higher *Bacteroides* RA
Larsen et al (2010), Wu et al. (2010), Qin et al. (2012), Karlsson et al. (2013), Sedighi et al. (2017)	• Higher RA of several *Lactobacillus* species • *Lactobacillus* RA positively correlated with PG levels
Larsen et al. (2010)	• Lower RA of Clostridia
Zhang et al. (2013)	• Higher RA of Clostridia

RA, relative abundance

Chapter Three: Objectives and Hypothesis

KNOWLEDGE GAP

Despite the aforementioned studies, there are still major gaps in knowledge. When broken down by race and ethnicity, the rate of diabetes in Hispanics is relatively high at 12.1%, compared to 7.4% in non-Hispanic Whites.[11] Causes of high T2DM prevalence in Hispanics include disparities in income, education, and access to healthcare, as well as genetic predisposition to obesity and insulin resistance.[39] More recently identified is the potential role of the gut microbiome in T2DM risk in Hispanic Americans. Ross, et al. compared the gut microbial composition between Hispanics in South Texas and the Human Microbiome Project (HMP) and found significant taxonomical differences between the two groups.[40] Notably, the differences seen between this Hispanic cohort, 87.3% of whom did not have T2DM, and the HMP were similar to the differences seen in previous studies that compared controls to subjects with T2DM.[41] Given this information, further investigation of the gut microbiome as a potential predisposing factor for T2DM in the Hispanic population is needed. Additionally, no studies discuss how baseline characteristics other than T2DM status might have contributed to their findings regarding gut microbiome composition. There is a need for studies that examine not only gut microbiome composition, but also differences in baseline characteristics between groups. Finally, conflicting results regarding diversity necessitate further investigation of gut microbial diversity differences between groups with and without T2DM.

The majority of the San Antonio, TX population is Hispanic, 90% of whom are of Mexican origin.[42] Among Bexar County residents, Hispanics represent the highest risk population with a diabetes rate of 13.1%, compared to both African Americans (12%), and Caucasians (8%).[43] Therefore, our research group is in an ideal position to be able to

examine the gut microbiomes of Mexican Americans with and without T2DM and address these gaps in knowledge.

OBJECTIVE 1:

The primary objective of this study was to compare the diversity of the gut microbiome between Mexican Americans with and without T2DM.

Hypothesis 1

Gut microbiome alpha diversity is significantly different between Mexican Americans with and without T2DM.

OBJECTIVE 2:

Evaluate gut microbiome compositional differences between Mexican Americans with and without T2DM.

Hypothesis 2

Gut microbiome composition is different between Mexican Americas with and without T2DM.

OBJECTIVE 3:

Identify demographic and other patient characteristics associated with gut microbial diversity.

Hypothesis 3

Patient characteristics, including age and BMI, are associated with gut microbial diversity.

Chapter Four: Methods

STUDY DESIGN

This was a cross-sectional study of volunteers from San Antonio, TX and surrounding areas from June 2017 to July 2018. This study was approved by the Institutional Review Board at UT Health San Antonio and funded by the Research to Advance Community Health (ReACH) Pilot Grant Program.

Subjects were recruited using newspaper advertisements in the *San Antonio Express-News*, *Southside Reporter*, and *Conexion*. Flyers were also placed in the Medical Arts and Research Center (MARC) in the South Texas Medical Center.

Those interested in participating called the research team, and a research team member pre-screened subjects using a detailed questionnaire designed to exclude patients taking certain medications and with certain disease states (Appendix). If participants successfully completed pre-screening, their contact information was given to the staff at the First Outpatient Research Unit (FORU) and the MARC to schedule their research visit.

STUDY POPULATION

Subjects were included if they were at least 18 years old and self-identified as Mexican American. Subjects were excluded if they had a history of prior gastrointestinal surgery altering the anatomy of the gastrointestinal tract or certain medication use. Medication use that warranted exclusion included (1) chronic daily use of any medications meant to alter gastrointestinal secretory or motor function (e.g., prokinetic agents, narcotic analgesics, laxatives, anticholinergics, anti-diarrheals) and (2) use of antibiotics, gastric-acid suppressing medications, or probiotics in the previous two months. Subjects were divided into groups based on T2DM status. Subjects were considered to have T2DM if they

had been previously diagnosed with T2DM and were currently receiving active treatment for diabetes. An overview of the study design can be found in Figure 4.

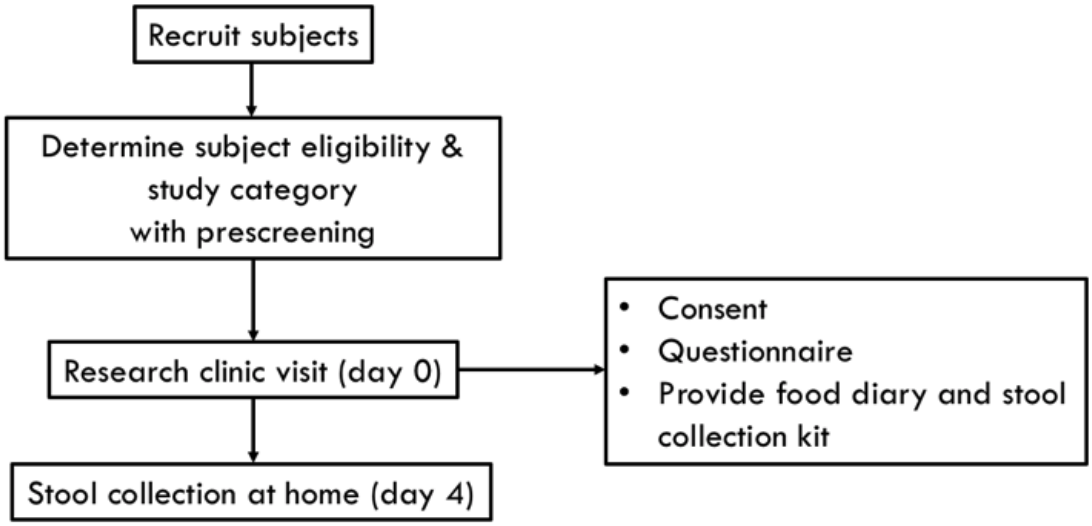

Figure 4: Overview of Study Design

DATA COLLECTION

Data collection was performed at the FORU at the MARC. Subjects attended a single visit where they filled out a demographic and health questionnaire. A list of data collected can be found in Table 4. Subjects were provided with a three-day food diary and stool sample collection kit. Subjects were instructed to fill out the food diary for the following three days and collect a stool sample on the fourth day to be sent back to our research team.

Table 4: Data Collected from Subjects

Age	Approximate income	Other conditions
Height	Current employment status	Medications
Weight	Tobacco use	Time since T2DM diagnosis
BMI	Alcohol use	HbA1c
Sex	Comorbidities (see Appendix for full list)	FBG
Highest level of education		

SAMPLE PROCESSING AND SEQUENCING

Stool samples were stored at -80 degrees C until sequencing. Microbiome Insights performed DNA extraction, sequencing, and analysis for our study. DNA was extracted from specimens using MoBIO PowerMag Soil DNA Isolation Bead Plate and KingFisher™ robot.

Bacterial 16S rRNA genes were PCR-amplified using primers targeting the V4 region. Primers were comprised of Illumina adapters, an 8-nucleotide index sequence, a 10-nucleotide pad sequence to prevent hairpin formation, and a gene-specific primer. Amplicons were sequenced using the Illumina MiSeq 300-bp paired-end kit (v.3).

Taxonomical classifications were denoised, taxonomically classified using the Greengenes v. 13_8 database, and clustered into 97%-similarity operational taxonomic units (OTUs) using Mothur Software package (v. 1.39.5).

HEI SCORE CALCULATIONS

HEI scores were calculated using subjects' three-day food diaries. Total calorie intake was estimated using the United States Department of Agriculture Food Composition Databases.[44] We assigned points based on the HEI-2015 scoring system, which is the most recent rendition.[45] Higher intake of adequacy components per 1000 kcal resulted in a higher

HEI score. Adequacy components included total fruits, whole fruits, total vegetables, greens/beans, whole grains, milk/dairy, total protein foods, seafood/plant proteins, and polyunsaturated and monounsaturated fatty acids to saturated fatty acids ratio. Higher intake of moderation components resulted in a lower HEI score. Moderation components included saturated fats, refined grains, sodium, and added sugars.

DATA AND STATISTICAL ANALYSES

Baseline characteristics were compared using JMP 14.0.0(R) (SAS Institute, Cary, NC, USA). Wilcoxon rank sum test was used for non-normal parametric data, chi-square test was used for nominal data, and Fischer's exact test was used when expected counts were less than five.

Objective 1

Alpha diversity was estimated with the Shannon index on raw OTU abundance tables after filtering out contaminants. The significance of diversity differences was tested using Wilcoxon rank sum.

Objective 2

Beta diversity was measured using Bray-Curtis indices and visualized using PCoA. OTUs were excluded if they occurred in fewer than 10% of samples with a count of less than 3.

Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) with treatment group as the main fixed factor and using 4,999 permutations for significance testing.

Objective 3

Subject characteristic comparisons were calculated using the Wilcoxon rank sum test. The impact of age and obesity on beta diversity was evaluated using PERMANOVA.

Chapter Five: Results

COHORT DESCRIPTION

The study was comprised of 37 subjects, 14 with T2DM and 23 without diabetes. All subjects self-identified as Mexican American. The median age (IQR) was 59 years (48-68), and 27 (73%) were female. Overall, participants were overweight, and about half (46%) had hypertension. Rates of other comorbidities can be found in Table 5.

Table 5: Baseline Characteristics

Characteristic	All subjects (N = 37)	Diabetes (n = 14)	No diabetes (n = 23)	p-value
Age, median (IQR), years	59 (48-68)	68 (59-72)	55 (38-61)	0.0032
Female, no. (%)	27 (73)	9 (64)	18 (78)	0.4537
BMI [*], median (IQR), kg/m^2	28.7 (26.6-34)	30 (26-36)	28 (27-31)	0.4653
Metformin, no. (%)	12 (33)	12(86)	0 (0)	≤ 0.0001
Sulfonylurea, no. (%)	3 (8)	3 (21)	0 (0)	0.0122
GLP-1 RA, no. (%)	3 (8)	3 (21)	0 (0)	0.0122
Insulin, no. (%)	2 (5)	2 (14)	0 (0)	0.0435
HMG-CoA Reductase inhibitor[+]	10 (28)	8 (57)	2 (9)	0.0016
ACEI/ARB[^]	11 (32)	8 (57)	3 (15)	0.0092
Beta-blocker[^]	5 (15)	3 (21)	2 (10)	0.3584
Diuretic	2 (5)	0 (0)	2 (14)	0.0435
Highest level of education, no. (%)				0.2893
High school or equivalent	8 (22)	2 (14)	6 (26)	
Some college, no degree	14 (38)	8 (57)	6 (26)	
Associate's degree	6 (16)	3 (21)	3 (13)	
Bachelor's degree	4 (11)	0 (0)	4 (17)	
Master's degree	1 (3)	0(0)	1 (4)	
Employment status, no (%)				≤ 0.0001
Retired	15 (41)	11(79)	4 (17)	
Employed for wages	17 (46)	1 (7)	16 (70)	
Out of work/looking for work	5 (14)	2 (14)	3 (13)	
Hypertension, no. (%)	17 (46)	9 (53)	5 (25)	0.0793
Dyslipidemia, no. (%)	10 (27)	5 (36)	5 (22)	0.3574
History of MI, no. (%)	1 (3)	1 (7)	0 (0)	0.3784
History of cancer, no. (%)	1 (3)	1 (7)	0 (0)	0.3784
Depression, no. (%)	1 (3)	1 (7)	0 (0)	0.3784
IBS, no. (%)	1 (3)	0 (0)	1 (4)	1.0000
HEI score, median (IQR)	53.5 (42.7-66.6)	62.0 (60.0-65.8)	48.5 (40.0-68.4)	0.2340
Household income, dollars, median (IQR)	24,000 (4,850-55,000)	25,500 (1,275-56,250)	24,000 (8,400-60,000)	0.7419
Mexico birth, no. (%)				
Subjects	3 (8)	2 (14)	1 (4)	0.29
Parents	9 (24)	3 (21)	6 (26)	0.7473
Grandparents	22 (59)	8 (57)	14 (61)	0.8230

IQR, interquartile range; BMI, body mass index; GLP-1 RA, glucagon-like peptide-1 receptor agonist; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; MI, myocardial infarction; IBS, irritable bowel syndrome; HEI, healthy eating index scores

*BMI not reported by one subject

[+]One subject excluded for reporting "cholesterol medication"

[^]Three subjects excluded for reporting "blood pressure medication"

Subjects with diabetes were older than those without diabetes (68 versus 55 years; p=0.0032). Rates of disease of the cardiovascular system (e.g. hypertension, dyslipidemia, and history of myocardial infarction [MI]) were not significantly different between groups; however, rates were numerically higher in those with T2DM. Twelve of the 14 subjects with diabetes (86%) took metformin.

OBJECTIVE 1: ALPHA DIVERSITY

There was no significant difference in Shannon diversity between subjects with and without T2DM, though subjects with T2DM had a slightly numerically lower alpha diversity (3.26 versus 3.31; p=0.341) (Figure 5).

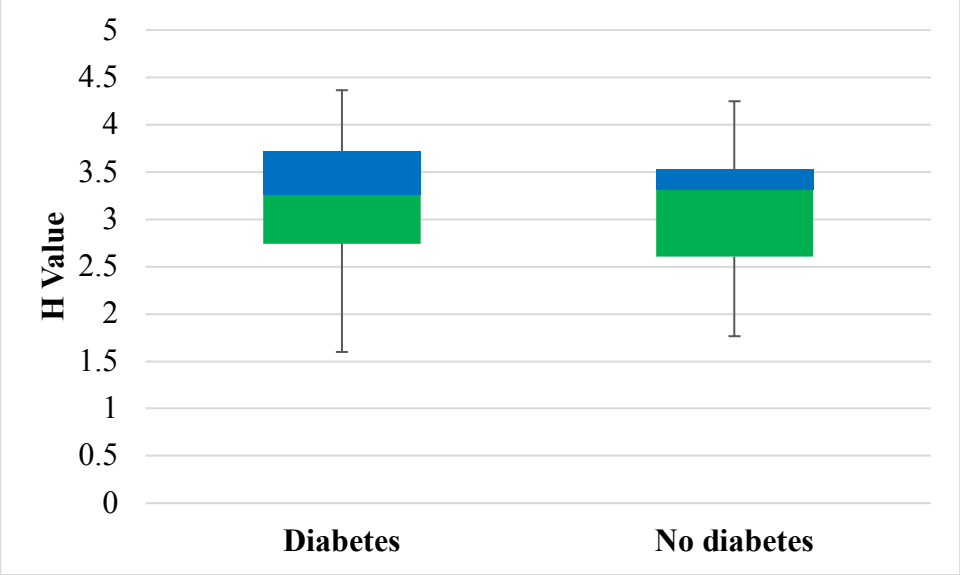

Figure 5: Shannon Diversity by Diabetes Status

OBJECTIVE 2: MICROBIAL COMPOSITION

The most dominant phyla for both groups were Bacteroidetes (56% in T2DM, 51% in non-T2DM, p=0.17), followed by Firmicutes (32% in both groups), and Proteobacteria (4% in both groups) (Figure 6).

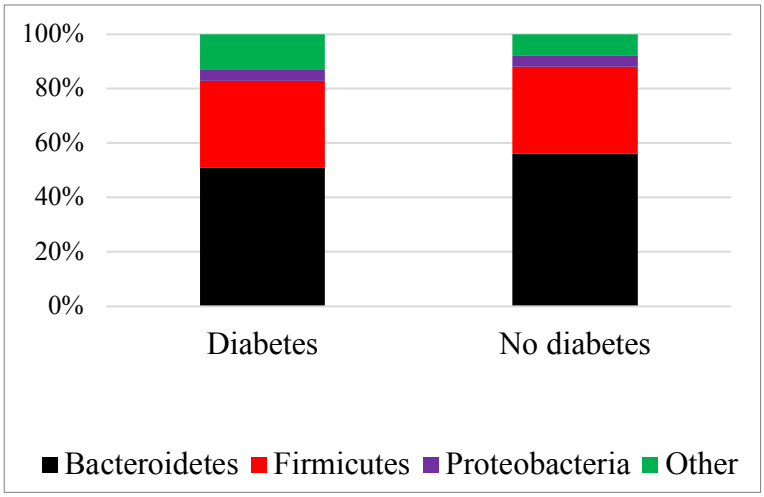

Figure 6: Dominant Phyla

The lower RA of Bacteroidetes resulted in a higher Firmicutes to Bacteroidetes ratio in subjects with T2DM (Table 6).

Table 6: Firmicutes to Bacteroidetes Ratio

	Diabetic	**Non-diabetic**
Firmicutes to Bacteroidetes ratio	0.637:1	0.507:1
Firmicutes RA	32%	32%
Bacteroidetes RA	51%	56%

RA, relative abundance

There was a significant difference in the RA of 16 OTUs between groups. Figure 7 depicts the microbiome taxa differences by T2DM status and BMI category.

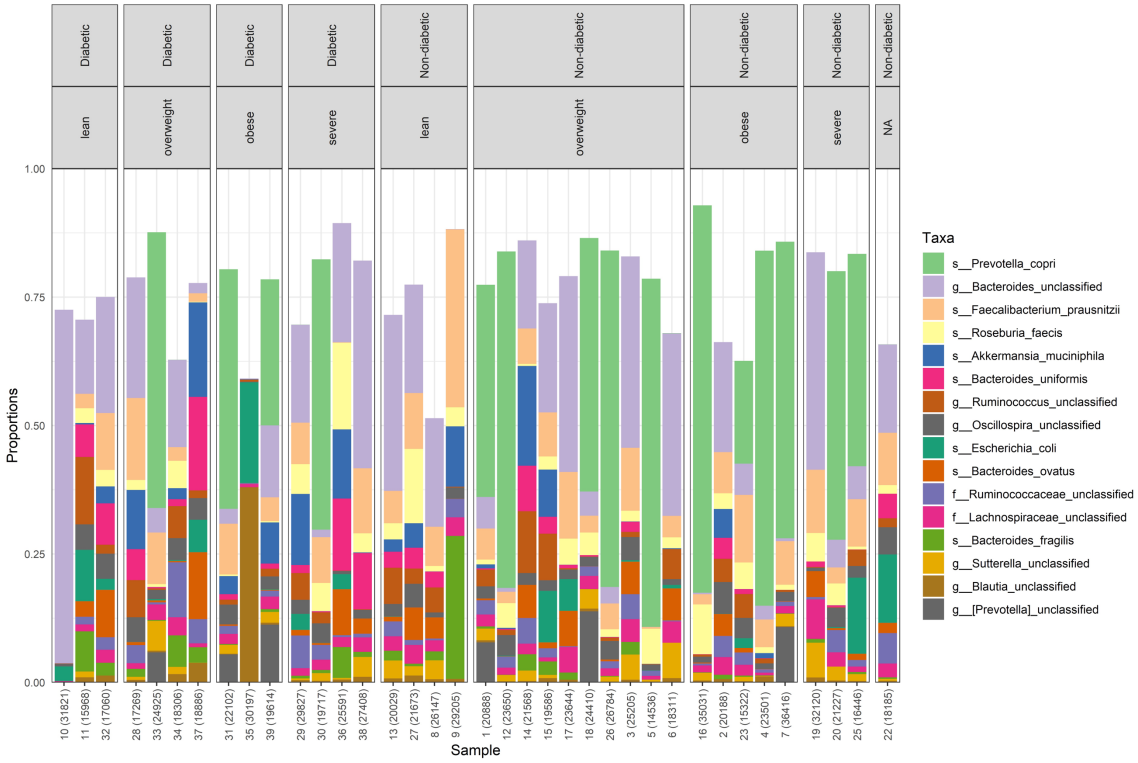

Figure 7: Relative Abundance by Species

When the RA of different genera were compared between groups, the proportion of *Streptococcus* was significantly higher in the subjects without T2DM (p=0.048). Additionally, subject 35 had a high RA of *Blautia*, as shown in Table 7.

Table 7: *Blautia* Median RA

Group	RA
Subject 35	38%
T2DM	0.7%
Non-T2DM	0.7%

OBJECTIVE 2: BETA DIVERSITY

There were no significant differences in beta diversity between subjects with and without T2DM as measured by PERMANOVA (p = 0.20) (Figure 8).

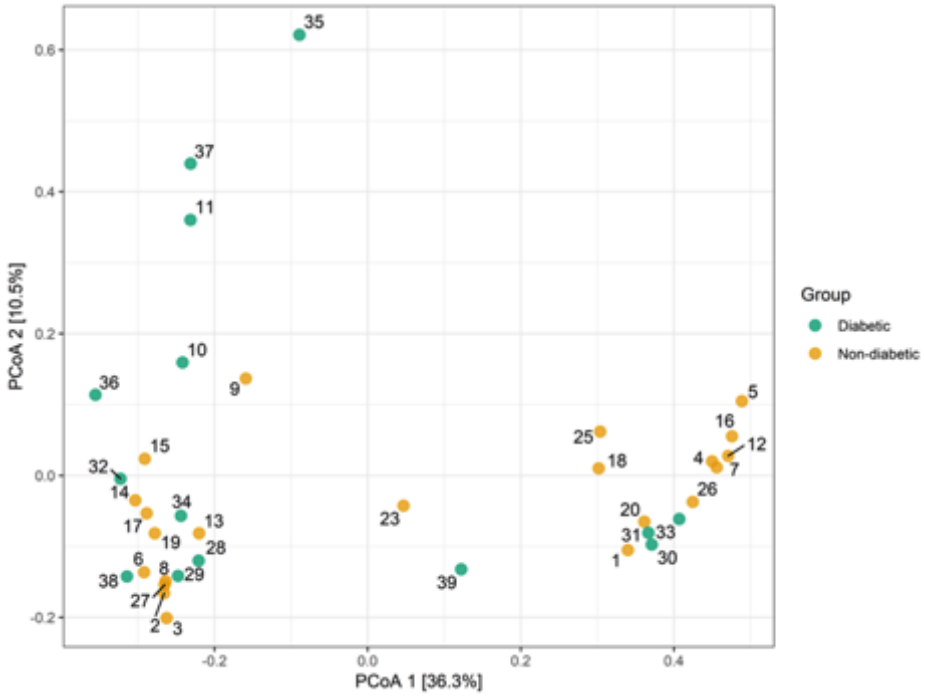

Figure 8: Microbiome Similarity

No strong associations are apparent by disease status; however, two distinct clusters are apparent in the lower left and lower right quadrants. Generally, there are more T2DM points on the left side of the PCoA. When HEI scores were calculated and assigned to each point, clustering was not significant (p=0.4962).

The most distinct coordinate is subject 35, who is obese and has diabetes. This subject was the only subject taking a tricyclic antidepressant.

OBJECTIVE 3: SUBJECT CHARACTERISTICS AND MICROBIAL DIVERSITY

Beta diversity was not significantly different by age although there was a scatter of older individuals toward the top left of the PCoA (Figure 9).

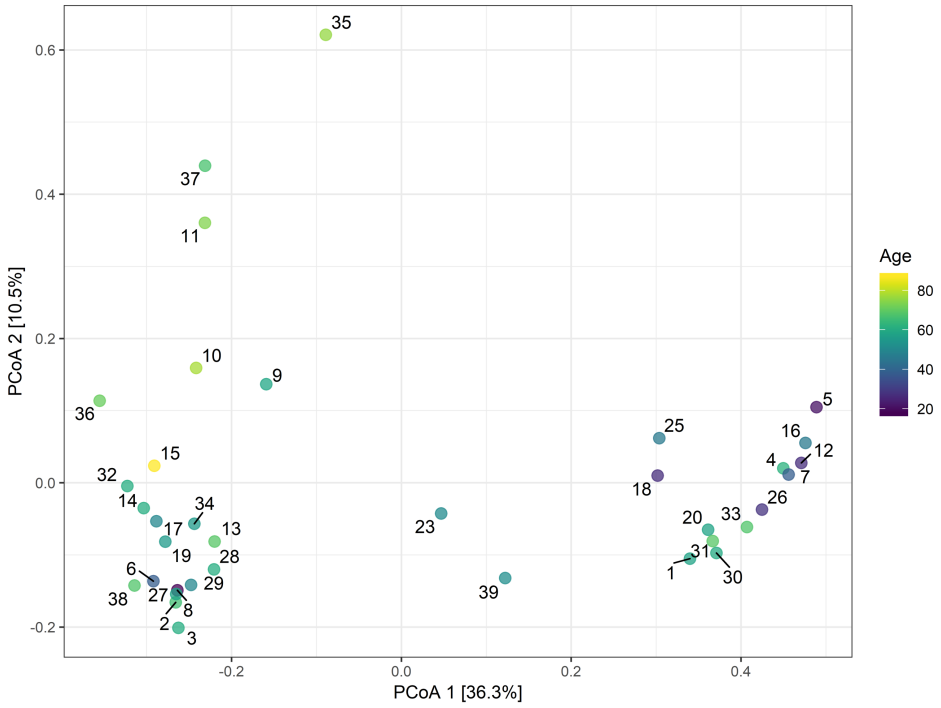

Figure 9: Microbiome Similarity by Age

Beta-diversity was not significantly different by BMI (Figure 10). However, there is a high degree of similarity between several high BMI subjects: 20, 25, and 30.

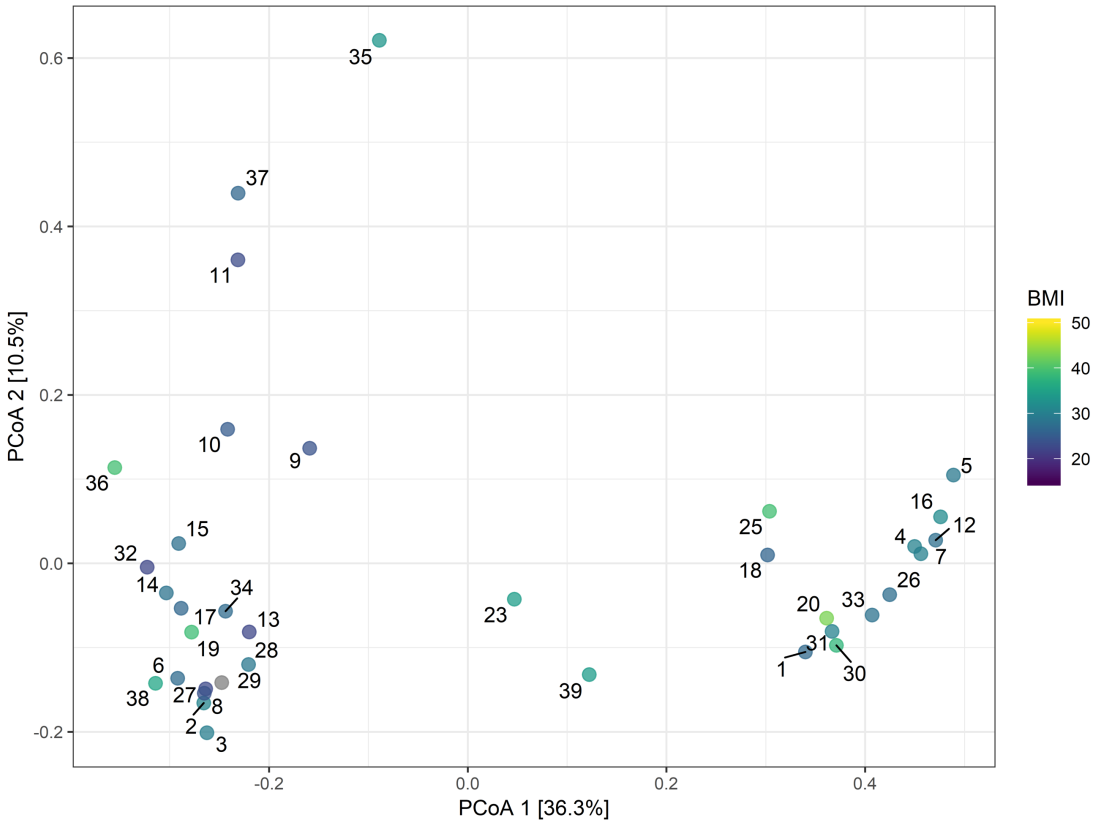

Figure 10: Microbiome Similarity by BMI

Furthermore, PCoA coordinates were not associated with HEI scores (p=0.49) or metformin use (p=0.7670).

Chapter Six: Discussion

OBJECTIVE 1

Our study found no difference in alpha diversity between Mexican Americans with and without T2DM (Figure 5). As mentioned previously, no prior studies examined gut microbial composition difference between Hispanics with and without T2DM. One study included 15 (31%) Hispanic subjects, but the majority of subjects were white (57%).[21] However, this study's findings agreed with our own in that alpha diversity was slightly, but not significantly, lower in prediabetic and T2DM subjects (5.26 and 5.21, respectively) compared to non-T2DM subjects (5.46).

Although alpha diversity has been shown to be a marker of multiple diseases, including obesity[46,47], colorectal cancer[33], and type 1 diabetes[29], multiple gut microbiome studies in T2DM demonstrated that alpha diversity is not significantly different between subjects with and without T2DM.[19,22] These studies have not provided rationale as to why there is no difference in alpha diversity. One study by Zhang, et al. determined that alpha diversity was negatively correlated with insulin resistance.[24] It is unclear why alpha diversity results are so variable. Multiple factors can contribute to gut diversity, including diet and medications.[48,49] Our study collected the data necessary to examine the influence of diet and medications on alpha diversity. For example, from subject food diaries, we calculated HEI scores. HEI scores are a validated method of quantifying diet quality that offers a less biased measurement than other methods, such as food-frequency questionnaires.[50] HEI scores were not significantly different between groups, with a median of 62 in those with T2DM versus 48.5 in those without T2DM. Though not significantly different, subjects with T2DM had a numerically higher HEI score, indicating overall superior diet. It is difficult to interpret the clinical significance of this 13.5 point

difference in the context of our study because previous studies have used HEI scores for stratifying subjects, while our study treated HEI scores as a confounding variable. It is possible that differences in HEI contributed to greater than expected bacterial diversity in T2DM subjects. Interestingly, the national average HEI score is 59, which is closer to the scores of our T2DM subjects compared to those without T2DM.[51]

Another major difference that could have contributed to lack of alpha diversity differences between groups is medication usage, specifically metformin, which is considered first-line therapy for T2DM according to the American Diabetes Association guidelines.[52] Metformin has been shown to increase gut microbial diversity compared to subjects with T2DM not on metformin.[53] The aforementioned studies did not report subjects' medication usage; therefore, these studies were not able to determine whether metformin usage contributed to the lack of differences seen in alpha diversity between groups.[19-24] Conversely, in our study 86% of subjects with T2DM took metformin compared to 0% in the non-T2DM subjects. This high rate of metformin use in those with T2DM could have led to increased alpha diversity in that group, resulting in similar alpha diversities between groups.

OBJECTIVE 2

The microbial composition was significantly different between groups. Most of the changes observed in this study did not support findings from previous studies (Table 8). For example, our study found no significant differences in the RA of *Lactobacillus*, *Bacteroides*, *Prevotella*, Clostidia, *Bifidobacterium*, or *Roseburia*.

Table 8: Microbial RA Compared to Other Studies

References	**Microbiota in subjects with T2DM – other studies**	**Microbiota in subjects with T2DM – our study**
Wu et al. (2010), Sedighi et al. (2017)	↓ *Bifidobacterium*	↔ *Bifidobacterium*
Larsen et al. (2010), Lambeth et al. (2015)	↓ Firmicutes	↔ Firmicutes ↑ Firmicutes:Bacteroidetes ratio
Larsen, et al. (2010), Qin et al. (2012), Karlsson et al. (2013)	↓ *Roseburia*	↔ *Roseburia*
Larsen et al. (2010), Zhang et al (2013)	↑ *Prevotella*	↔ *Prevotella*
Wu et al. (2017)	↓ *Prevotella*	
Zhang et al. (2013)	↓ *Bacteroides*	↔ *Bacteroides*
Wu et al.	↑ *Bacteroides*	
Larsen et al (2010), Wu et al. (2010), Qin et al. (2012), Karlsson et al. (2013), Sedighi et al. (2017)	↑ *Lactobacillus*	↔ *Lactobacillus*
Larsen et al. (2010)	↓ Clostridia	↔ Clostridia
Zhang et al. (2013)	↑ Clostridia	

↑, increase; ↓, decrease; ↔, no significant difference

Our findings regarding the Firmicutes to Bacteroidetes ratio conflicts with findings from previous studies, including the study by Lambeth, et al., which included 15 (31%) Hispanic subjects.[21] Lambeth, et al. found a lower RA of Firmicutes in subjects with T2DM (34.4%) compared to those without (39.7%), and Bacteroidetes RA was about the same in both groups (Table 9). This resulted in a lower Firmicutes to Bacteroidetes ratio in subjects with T2DM, which conflicts with our results. Of note, Bacteroidetes RA was the main driver of the Firmicutes to Bacteroidetes ratio in our study, whereas Firmicutes RA was the main driver in the study by Lambeth, et al.

Table 9: Firmicutes to Bacteroidetes Ratio in Previous Study

	Our study		Lambeth, et al.	
	Diabetic	**Non-diabetic**	**Diabetic**	**Non-diabetic**
Firmicutes to Bacteroidetes ratio	0.637:1	0.507:1	0.64:1	0.74:1
Firmicutes RA	32%	32%	34.4%	39.7%
Bacteroidetes RA	56%	51%	53.9%	53.5%

Increases in this ratio have been implicated in increased energy harvest and obesity.[54] Though our subjects with and without T2DM did not have statistically different BMIs, the T2DM group had a numerically higher median BMI of 30, which is classified as obese. The non-T2DM group had a median BMI of 28, which is considered overweight. Thus, the increase in the Firmicutes to Bacteroidetes ratio may indicate that the ratio is more related to BMI and obesity as opposed to diabetes status. Another possible explanation is that the Firmicutes to Bacteroidetes ratio is related to T2DM control.

Streptococcus genus had a significantly higher RA in non-T2DM subjects compared to subjects with T2DM. *Streptococcus* has been associated with atherosclerotic cardiovascular disease (ASCVD), hypertension, and enhanced thrombotic risk.[55-57] It is therefore surprising that *Streptococcus* was enriched in our non-T2DM subjects as they had overall lower rates of cardiovascular-related diseases, and T2DM itself is a risk factor for cardiovascular disease. One possible explanation for this is the microbiome modulatory effects of metformin. The groups were similar except for diabetes status, and the majority of our diabetic subjects were on metformin. Metformin use could have resulted in a microbiome that is less like that of a person with ASCVD. In addition, differences in diet could have contributed to the observed difference in *Streptococcus* RA.

Contrary to previous studies, there was no association between beta-diversity and T2DM status.[20,22] This is likely because the control groups in previous studies were

healthier in general, whereas the non-T2DM subjects in our study had high rates of cardiovascular-related disease and a high median BMI, making their overall health more similar to the T2DM group. Furthermore, as mentioned previously, metformin demonstrates microbiome-modulating effects in T2DM.[53] Those taking metformin have increases in butyrate-producing bacteria. Although our study did not show significantly higher RA of specific butyrate-producing species, such as *Roseburia spp.*, we did observe an overall increase in Firmicutes, many of which are butyrate-producers.[18]

The most visually distinct microbiome on the PCoA was that of subject 35, who was allowed into the study while taking a tricyclic antidepressant because there are currently no microbiome studies examining tricyclic antidepressants' direct effects on the gut microbiome composition. When analyzed, subject 35's gut microbiome demonstrated a high RA of *Blautia*. This subject was obese, so these findings are consistent with previous studies which demonstrated this genus's association with high BMI.[57,58] However, tricyclic antidepressant use may also play a role in this subject's gut microbial composition. There is an established relationship between the gut and brain, referred to as the gut-brain axis, wherein the gut microbiota and the brain utilize bidirectional signaling to maintain homeostasis.[59] Selective serotonin reuptake inhibitors (SSRIs), another class of serotonergic antidepressants, have been shown to impact gut microbiome composition.[60] There are currently no studies evaluating the effects of tricyclic antidepressants on the gut microbiome. Our findings suggests that tricyclic antidepressants, like SSRIs, may impact the gut microbiome composition.

OBJECTIVE 3

Beta diversity was not significantly different between subjects when analyzed by subject age or BMI. This is surprising as prior studies have identified these to be major mediators of the gut microbiome. As noted previously, BMI had been associated with differences in the Firmicutes to Bacteroidetes ratio, and weight loss results in changes in microbial composition.[61] Aging has been associated with decreased bacterial diversity, a decrease in SCFA-producing bacteria, and compositional differences that predispose hosts to pathogen invasion.[62] The lack of significance seen in beta-diversity when compared by BMI and age indicates that microbial similarity between subjects is complicated and multi-faceted. Our subjects' baseline characteristics likely played a role in these results. Our subjects were well-matched with respect to comorbidities, BMI, and diet.

One major difference between our study and previous studies is that we collected and reported a plethora of baseline and demographic characteristics. Previous studies rarely reported patient characteristics and demographic information, which limits the ability to interpret their results in the context of other comorbidities. Of the two studies that reported baseline data, the subjects were healthier overall, with lower BMIs and fewer comorbidities compared to those in our study.[20,24]

The similar health status between our cohorts may also explain other discrepant findings. For example, as mentioned previously, contrary to previous findings, the T2DM group in our study had a higher RA of Firmicutes. This may indicate that the ratio is more related to BMI and obesity as opposed to diabetes status.

STRENGTHS

This was the first study to compare the gut microbiome composition between diabetic and non-diabetic subjects in a completely Mexican American population. Mexican Americans have been underrepresented in gut microbiome studies of diabetes despite having higher diabetes prevalence compared to most other races and ethnicities.

Additionally, we collected extensive background and health information, including medication use, height, weight, and comorbidities, which is helpful when interpreting study results, as all of those characteristics can influence and be influenced by the gut microbiome.

Finally, we compared two well-matched groups; both groups had high median BMIs and similar rates of cardiovascular comorbidities. To date, most T2DM gut microbiome studies have compared healthier subjects with lower BMIs and no reported comorbidities. While this allowed investigators to focus on the relationship between the gut microbiome and diabetes alone, a relationship also exists between the gut microbiome and obesity and other comorbidities. Because the comparator group has these comorbidities, our study offers valuable insights into the kind of dysbiosis connected specifically to diabetes even when other comorbidities are present, as they often are in clinical practice.

LIMITATIONS

There are several limitations to the study which stem mostly from the fact that it was a small pilot study. For example, the study may not have been powered to detect the differences in microbial RA identified in previous studies. However, despite this small sample size, we were able to identify several novel differences between groups.

Another limitation was that all demographic and health information was self-reported by the subjects. Up to one-third of all people with diabetes are not diagnosed, so it is possible that some of our non-diabetic subjects had diabetes. Furthermore, comorbid diseases, height, weight, and medications might have been inaccurately reported.

Finally, we could not control for all microbiome mediators. Although we were able to show that the groups were well-matched with respect to diet, comorbidities, and BMI, there are too many mediators of the microbiome to be able to account for them all.

CONCLUSIONS AND FUTURE RESEARCH

Alpha and beta diversity were not significantly different between diabetic and non-diabetic Mexican American subjects; however, microbial composition was significantly different between groups.

The results of this study offer valuable insight into how scientists can target the gut microbiome of individuals who have T2DM and other comorbidities. Additionally, having knowledge of how the gut microbiomes of Mexican Americans differ from those examined in previous studies will allow researchers to more accurately target individuals' gut microbiomes to prevent or treat T2DM.

Further, several interesting findings from this study could stimulate further research. For example, the high RA of *Streptococcus* in non-diabetic subjects with lower rates of diseases that predispose one to atherosclerotic cardiovascular disease warrants further investigation. Also, tricyclic antidepressant effects on the gut microbiome is another area thus unstudied that could be examined. Finally, it is possible that metformin modulates multiple facets of metabolic health through its effects on the gut microbiome. Comparative studies are needed to examine the gut microbiome pre- and post-metformin initiation.

The next step in the analysis of this data set is to compare it to HMP data to determine the impact of Mexican American ethnicity on the gut microbiome.

Appendix: Data Collection Sheet

Age: **Height:** **Weight:** **Sex:**

Country of birth (please circle one for each:

Self:	United States	**Parents:**	United States	**Grandparents:**	United Sates
	Mexico		Mexico		Mexico
	Other		Other		Other

Highest level of education completed (please circle one):

No schooling
Some high school, no diploma
High school graduate or equivalent (ex. GED)
Trade/technical/vocational training
Some college, no degree
Associate degree
Bachelor's degree
Master's degree
Doctorate degree (ex. PhD, MD, PharmD)

Approximate household income: $ _____

Current employment status (circle one):

Employed for wages
Out of work and looking for work
Out of work but not currently looking for work
Homemaker
Military
Student
Retired
Unable to work

Social History:

Do you use either of the following in any quantity (please circle no or yes)?

Tobacco	No	Yes	Approximate quantity per week:
Alcohol	No	Yes	Approximate quantity per week:

Medical History:

Please circle any medical condition you currently have or have a history of:

High blood pressure	Kidney disease	Peptic ulcer disease
High cholesterol	Cancer	Liver disease
Heart attack	HIV or AIDS	Inflammatory bowel disease
Heart failure	Anxiety	Irritable bowel syndrome
Vascular disease	Depression	
Stroke	Dementia	
Diabetes	COPD	

Please list all other chronic medical conditions:

Medication History:

Please list all medications, including prescription, over-the-counter, and herbal medications used daily or in the past 2 months:

**** For participants with diabetes ****

Approximate time since first diabetes diagnosis (if known): _____
Last hemoglobin A1c value (if known): _____
Last fasting glucose value (if known): _____

Glossary

ASCVD	Atherosclerotic cardiovascular disease
BMI	Body mass index
FORU	First Outpatient Research Unit
GLP-1	Glucagon-like peptide 1
GPCR	G-protein coupled receptor
HEI	Healthy Eating Index
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
LPS	Lipopolysaccharide
MARC	Medical Arts and Research Center
OTU	Operational taxonomic unit
PCoA	Principle coordinate analysis
PCR	Polymerase chain reaction
RA	Relative abundance
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acids
SSRI	Selective serotonin reuptake inhibitor
T2DM	Type 2 diabetes mellitus
TLR4	Toll-like receptor-4

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